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A Survey of Analytical Procedures for Traces of N-Nitrosamines in Foods

APPLIED CHEMISTRY DIVISION : FOOD SECTION

Commission on Trace Substances

A SURVEY OF ANALYTICAL PROCEDURES FOR TRACES OF N-NITROSAMINES IN FOODS

The following survey is based on the Working Paper prepared by Dr A E Wasserman on behalf of IUPAC Applied Chemistry Division Food Section for a meeting arranged by the International Agency for Research on Cancer jointly with the Deutsches Krebsforschungszentrum in Heidelberg 13-15 October 1971 to discuss the analysis and formation of nitrosamines.

Dr Wasserman presented the paper to the meeting, which was also attended by Dr A J Collings, Secretary of IUPAC Food Section. Particular thanks are expressed for the cooperation of the many investigators who submitted their procedures and written comments at the invitation of the Trace Substances Commission of the Food Section and to its Commission's Smoke Constituents Sub-Commission. Personal communications in this way are acknowledged in the text by undated references to the correspondents. The membership of the Smoke Constituents Sub-Commission was Dr H Fischbach (Chairman), Dr A E Wasserman (Secretary), Dr C L Cutting, Prof. P P Dikun and Dr S J Kubacki. Thanks are also expressed to Mr J K Foreman and Mr J F Palframan for assistance in editing the survey following the meetings in Heidelberg.

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Chairman, Food Section

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Ender et al. (1964) reported the presence of N-nitrosodimethylamine in fish meal as the casual agent of hepatotoxic distress in sheep and mink. Since then the possibility of the occurrence of N-nitrosamines in human food supplies has become internationally recognised as a problem on which reliable analytical information is required to assess their potential hazard to human health. Although the N-nitrosamines form a class of compounds, there are differences in characteristics among the members and difficulties are encountered in analytical procedures. Indications to date are that the concentration of N-nitrosamines in foods, if present at all, is of the order of a few µg/kg. Recovery in good yield of such low concentrations of nitrosamines from foods of varying substances, quantitation and finally confirmation of the nitrosamines are the stages involved in the determination of these compounds. Each of these stages is a potential source of difficulty in the procedure.

A number of studies involving the analysis for N-nitrosamines have been reported in the literature. Each investigator, for the most part, developed his own procedures. Because earlier methods lacked the required specificity caution is necessary in interpreting the early findings both in terms of the nitrosamines identified and the concentrations reported. The Trace Substances Commission of the Food Section, International Union of Pure and Applied Chemistry, has recognised the need at the international level for an acceptable procedure for the estimation of traces of N-nitrosamines. Not one of the methods in the literature, however, appears to have been used by enough investigators to be considered as a basis for a collaborative assay. A survey of a number of reported methods has been prepared in an effort to clarify the situation. In the following outline various methods of carrying out the different stages of the analytical procedure are listed. Only the major points of the various reports were considered and the original references should be consulted for details.

OUTLINE OF PROCEDURES USED IN ANALYSIS FOR N-NITROSAMINES

EXTRACTION, CLEANUP AND CONCENTRATION

A - Distillation

1 - From alkali

a - Steam distil then concentrate by repeated distillation to half volume from 3N NaOH. (Sen et al. 1970; anon. 1969; Eisenbrand & Preussmann 1970; Ender & Céh 1967; Crosby*; Saxby*)

b - Digest product by refluxing with alcoholic KOH, then steam distil. (Howard et al. 1970; Fiddler et al. 1971; Howard et al. 1971)

2 - From acid

a - Distil from alkali first to avoid artifact formation at low pH. (Eisenbrand et al. 1970)

b - Steam distil directly from acid (anon. 1969)

* Personal Communication (1971)

3 - From neutral solution

a - Distil from neutral 20% NaCl solution first to avoid formation of artifacts - second distillation from 0.01N acid to remove basic materials (anon. 1970)

4 - Other

a - Distil from aqueous MeOH+5% NaCl and collect fraction boiling between MeOH and water. (Walters et al. 1970; Saxby*)

b - Vacuum distil slurry + 20% NaCl + potassium carbonate (Sen 1970; Telling et al. 1971)

B - Solvent Extraction

1 - Methylene chloride (CH_2Cl_2)

a - Extract with methylene chloride, remove basic components with 1% HCl wash (Neurath 1964)

b - Extract wheat flour with solvent (Heyns & Koch 1971)

c - Extract product with solvent, condense by distillation and nitrogen evaporation, then steam distil. (Kröller 1967)

d - Distillate extracted with methylene chloride (Serfontein & Hurter 1966; Saxby*; Howard et al. 1970; Telling et al. 1971)

e - From tobacco; dry, neutral methylene chloride extract is treated with pentane to precipitate contaminants (Neurath 1964)

f - distillate adjusted to pH 3 after hexane extraction, extract with methylene dichloride; extract washed with N sodium hydroxide (Fiddler et al. 1971)

2 - Heptane

a - Distillate extracted with heptane then washed with NaOH (Kröller 1967)

3 - Acetonitrile - heptane

a - Extract methylene chloride extract of product with acetonitrile - heptane to reduce lipid interference. (Eisenbrand et al. 1970 a; Eisenbrand et al. 1969)

4 - Ether

a - Extract with ether, remove ether and steam distil (van Ginkel)

b - Extract from cheese with ether, remove ether, steam distil, make alkaline with barium hydroxide and distil. (van Ginkel)

5 - Water

a - Extract product with water, precipitate proteins and basic compounds with phosphotungstic acid; steam distil after adding NaOH to 3M concentration. (Ender & Céh 1967)

C - Column Chromatography

a - Distillate passed through sulfonic acid-ion exchange column. Aqueous eluate extracted with methylene chloride to recover nitrosamines. (anon. 1969; Crosby*)

b - Polyamide - ion exchange: extract effluent with methylene chloride, transfer to pentane and run through alumina (Woelm basic, cationotrophic, activity grade I) (Sen et al. 1969)

c - Acid Celite 545 - concentrated extract placed on column, washed with pentane, transferred to methylene chloride (Howard et al. 1970; Fazio et al. 1971)

Note: Extraction with methylene chloride or polar solvents may lead to low nitrosamine recoveries (Kadar and Devik*)

* Personal Communication (1971)

D - Charcoal Adsorption

Nitrosamines are adsorbed from distillate or clarified homogenates. Excessive lipid concentration interferes with adsorption.

1 - Description

a - With boiling MeOH. (Walters et al. 1970)

b - By steam distillation. (Ender & Céh 1967)

DETECTION

A - Polarography

1 - Half-wave potential determined in 0.2N HCl solution. (anon. 1969; Heyns & Koch, 1970, 1971; Kadar & Devik*)

2 - To differentiate nitrosamines from artifacts (Walters et al. 1970)

a - pH adjusted to 8.4 with borax - nitrosamine wave disappears, new wave visible at lower potential.

b - UV irradiation in acid solution for 2 hours - nitrosamine wave disappears.

B - Spectrophotometry

1 - Direct: Separate on Sephadex LH 20 with methanol-water (1+1), read at 230-235 nm (pure nitrosamine solution only). (Eisenbrand et al. 1970 b)

2 - Colorimetric: Nitrosamine cleaved with HBr in glacial acetic acid. Free NO^+ diazotised with sulfanilic acid and N-(naphthyl-1)-ethylenediamine. Sensitivity: 0.114×10^{-7} mole gives absorption of 0.1; detection: in the 2-5 ppb range; average recovery of 16 or 17 nitrosamines 99.5%. (Eisenbrand & Preussmann 1970) After colorimetric determination of free NO^+ the corresponding amine can be derivatized, identified and measured.

3 - Reduction

a - to amine. Reduce with CuCl in conc.HCl. Distil from alkaline system and condense with 4'-nitro-4-azobenzoic acid in benzene. Remove excess reagent and separate by TLC on Silica gel G with chloroform. Extract spots with chloroform and read on spectrophotometer. Detection limit: 0.3-0.4 µg dimethylnitrosamine. (Kadar & Devik*)

b - to hydrazine. Reduce with Zn at pH 2.0, clean up and react with p-dimethylaminobenzaldehyde. Read at 230 or 330 nm. Sensitivity: 1-10 ppb. (Ender & Céh 1967)

C - Thin Layer Chromatography

1 - Used in many procedures for clean-up or for separation of nitrosamines. Silica gel G plates generally used and several developing solvents are listed. (Eisenbrand et al. 1970 c)

2 - For detection

a - Spray with sulfanilic acid and 1-naphthylamine

b - Spray with palladium chloride/and diphenylamine. (van Ginkel); Neurath 1964; Sen et al. 1969; Krüller 1967)

c - Spray with ninhydrin. (Sen et al. 1969; Krüller 1967)

d - Colored derivatives. (Neurath 1964; Eisenbrand & Preussmann 197 Kadar & Devik*)

* Personal Communication (1971)

e - Reduce with lithium aluminum hydride, clean up and react with 5-nitro-2-hydroxybenzaldehyde. Separate by TLC on Silica gel G (250 nm thick) with carbon tetrachloride-ethylacetate (19+1) Detection limit: 0.5 µg. Separated spots can be analyzed by gas chromatography.

D - Gas Chromatography

1 - Special Detectors

a - Nitrosamines oxidised to nitramines, then separated by TLC with trifluoracetic acid-50% H_2O_2 (5+4). Clean up and detect by gas chromatography using electron capture detector. Sensitivity: 16 pg nitrosamine. (Sen 1970)

b - Oxidise nitrosamines with peroxytrifluoracetic acid. (Reported for model systems only, Althorpe et al. 1970)

c - Reduce (no agent given) and couple with 1,1,1-trifluoracetyl-acetone. Separate by gas chromatography and detect with electron capture detector (anon. 1969)

d - Alkali Flame Ionization - has increased selectivity toward nitrogen-containing compounds. (1) Potassium chloride salt tip - sensitivity of assay: 10 ppb. (Howard et al. 1970); (2) Rubidium sulfate salt tip - limit of detectability: 5 ng, sensitivity of assay: 25 ppb. (Fiddler et al. 1971; Foreman and Palframan 1971)

e - Coulson conductimetric
Nitrosamines pyrolyzed to ammonia and dissolved in water, change in conductivity is measured. Highly selective for amines. Sensitivity: Nanogram level. (Rhoades & Johnson 1970; Essigman & Issenberg 1971; Foreman and Palframan 1971)

2 - General column procedures

a - 10% Carbowax 1540 on 80/100 mesh Gas Chrom Q in 100 cm column. Temperature programmed from 70° to 150° at 5°/min; used with Coulson detector (Rhoades & Johnson 1970)

b - 6% Reoplex 400 on 60/80 mesh Chromosorb W in 125 cm x 3 mm column at mesh Chromosorb W in 125 cm x 3 mm column at 48°. Flame ionization detector. (Sen et al. 1969)
Limits of detection and identification: 0.05 to 0.15 ppm.

c - Two columns in series; each 2m x 3mm i.d. (1) 5% SE 32 on 60/100 mesh Celite 545. (2) 15% Silicone Oil DC0 on 60/100 mesh Celite 545. Temperature: 200°. Thermal conductivity detector (Kröller 1967)

d - 1% SE 30 on acid washed, silanized Chromosorb G in 2 m x 2.7mm i.d. glass column. Temperature: 165°. (Kröller 1967)

e - 1% polyethylene glycol 4000 on acid washed 35/65 mesh Celite 545 in 50 m x 8 mm column. Temperature: 100°. Flame ionization detector. (Serfontein & Hurter 1966)

f - 15% Carbowax 1000 on 80/100 mesh Gas Chrom P in 1.19m x 12.5 mm glass column. Temperature: 100°. (DuPlessis et al. 1969)

g - Two columns in series. (1) 10% diethylene glycol adipate on acid washed, silanized 80/100 mesh Chromosorb W in 2m x 3mm o.d. Teflon column. (2) Mixture of copper (I) thiocyanate and acid washed, silanized Chromosorb W (10%) in 10 cm x 3 mm o.d. Teflon column. Temperature programmed from 50° to 180° at 5°/min. (Saxby 1970)

h - 10% Carbowax + 3% KOH on 100/200 mesh Gas Chrom P in 230 cm x 4mm i.d. glass column. Temperature programmed from 80° to 120° at 10°/min after initial hold at 80°. Alkali flame ionization detector. (Howard et al. 1970)

i - 15% Carbowax 20M-TPA on 60/80 mesh Gas Chrom P in 230cm x 3 mm o.d. column. Temperature: 115°. Alkali flame ionization detector. (Fiddler et al. 1971)

j - Several columns described: (1) 50 cm column Silicone Oil. Temperature: 60°, (2) 150 cm column containing Apiezon L. Temperature: 125° (van Ginkel); 150 cm column containing Carbowax 20M. Temperature: 150° (Petrowitz 1968)

k - Two columns, each 25m x 0.25 mm i.d. Ucon LB 550x + 1% KOH, temperature programmed from 45° to 150° at 5°/min. (2) Marlophen 87 CWH + KOH, temperature programmed from 45° to 125° at 5°/min. Flame ionization detector. Detection limit: 0.2-0.5 µg. Identification of peaks by mass spectrometry. Detection limit: 0.01 µg at ion source (Heyns & Koch, 1970; Heyns & Köper 1970)

l - Chromosorb 101 2m x 3 mm at 200°C (Foreman, Palframan & Walker 1970)

E - Mass Spectrometry

For final unambiguous characterisation of nitrosamines a combination of mass spectrometry and gas chromatography is often employed. (Heyns & Koch 1970; Heyns & Köper 1970; Crosby*; Telling 1971)

PROBLEMS IN NITROSAMINE ANALYSIS PROCEDURE

In view of the potential public health significance of the presence of N-nitrosamines in food, analytical procedures should record correctly and with known accuracy the nitrosamine content of the product under examination. Unfortunately, a number of reports have appeared that were carried out with analytical procedures of inadequate specificity and consequently in some instances naturally occurring substances and compounds produced during the procedure were reported as nitrosamines. Since observations concerning these problem areas have appeared, data obtained by the particular procedures should be carefully evaluated in relation to the stringent analytical criteria now known to be essential in determining nitrosamines.

Thin layer chromatography has frequently been used to separate the nitrosamines prior to identification. The compounds often are made visible by spraying with the reagents described by Preussmann et al. (1964); palladium (II) chloride-diphenylamine and the Griess reagent (sulfanilic acid-1-naphthylamine). However, Preussmann et al. (1964) also observed that the palladium chloride-diphenylamine reagent reacts with a number of other classes of compound e.g. unsaturated hydrocarbons, dicarbonic acids and compounds with α-unsaturated carbonyl groups. Any of these materials with R_f values similar to nitrosamines on thin layer plates could be erroneously identified as a nitrosamine. Preussmann et al. (1964) recommended that responses to both spray reagents should be positive before identification was made. Sen et al. (1969) identified nitrosamines on the basis of positive responses to both Griess reagent and ninhydrin By modifying the clean-up procedure they removed amines and yellow pigments present in fish and cheese that interfered in identification. Linoleic acid, α-tocopherol, xanthophyll and carotene also yielded spots that could be mistake for nitrosamines (Krüller 1967) and van Ginkel (1968) found propionic acid produced a spot on spraying with palladium (II) chloride-diphenylamine reagent the same R_f as N-nitrosodiethylamine. van Ginkel also reported extracting with ether from absorbent cotton and filter thimbles a number of compounds that gave the same purple color with the palladium (II) chloride reagent and had R_f value similar to those of known nitrosamines. In a quantitative procedure developed Daiber & Preussmann (1964) the N-nitrosamines are decomposed by UV light into

* Personal Communication (1971)

corresponding secondary amines and nitrite. The liberated NO^+ is determined with the sulfanilic acid 1-naphthylamine reagent. van Ginkel (1968) observed increased absorption values on irradiating demineralized water in a closed quartz cuvette. This did not occur with distilled water.

Pyrazines are often found in food products as a result of the thermal degradation of amino acids and sugars heated above 100°. These compounds interfere with the polarographic determination of nitrosamines, giving half-wave potentials similar to some of the nitrosamines (Heynes & Koch 1970, 1971). Pyrazines may also be mistaken for nitrosamines when gas chromatography is used for separation and tentative identification of these compounds.

Nitrosamines can be detected by electron capture gas chromatography and quantitated in the picogram range; however, oxidation of the N-nitrosamine to nitramine is not quantitative and may be variable (Sen 1970; Althorpe et al. 1970). The losses, in addition to losses in recovery of the nitrosamine from the food product, may be significant. Reduction of N-nitrosamines to hydrazines forms the basis of a number of techniques but quantitative conversion data are not yet available. Nevertheless the formation of electron capturing derivatives from hydrazines so formed offers a potentially sensitive and selective method of nitrosamine analysis.

The removal of nitrosamines from food products and subsequent clean-up are critical areas in the procedure. If extraction techniques are utilized it is important to ensure extraction of the nitrosamines from the product in high and known yield for subsequent quantitative analyses. In many procedures the product is first distilled or steam distilled from alkali, or from acid, and the distillate used for further processing. Warnings have appeared of the danger, at slightly acid pH values, of nitrosamine formation at this stage from the reaction of nitrites that may be present with amines formed by degradation. Reduction or complete removal of background material is important whether the final stage of the method is TLC, GC, or GC/MS. The µg/kg concentration of nitrosamines expected in foods are readily swamped by what are generally considered as trace contaminants and detection, identification or quantitation becomes extremely difficult. Column chromatography, TLC and liquid-liquid extractions are often used in the clean-up procedure; recovery checks after these treatments are essential in assessing overall losses. Loss of nitrosamines also occurs during concentration procedures and evaporation of solvents with nitrogen should be carried out with care. In particular the solvent must be dry to eliminate azeotrope formation with water.

The procedures and problems outlined above refer to those nitrosamines which are volatile in steam or are readily extractable by organic solvents. These comprise essentially the lower alkyl and certain heterocyclic nitrosamines such as N-nitrosopyrrolidine and N-nitrosopiperidine. Very little is known regarding the possible formation of non-volatile or inextractable N-nitroso compounds. This area requires intensive study but it awaits better definition of the problem in terms of which nitrosamines might be formed and suitable techniques for their isolation and purification prior to determination.

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